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(21) International Application Number: PCT/US98/21444 (22) International Filing Date: 9 October 1998 (09.10.98) (30) Priority Data: 08/948,276 9 October 1997 (09.10.97) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 5th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventor: ERNST, Joel, D.; University of California, San Francisco, P.O. Box 0868, San Francisco, CA 94143-0868 (US). (74) Agent: OSMAN, Richard, Aron; Science & Technology Law Group, 75 Denise Drive, Hillsborough, CA 94010 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: GFP-ANNEXIN FUSION PROTEINS (57) Abstract Bifunctional green fluorescent protein (GFP)-annexin fusion proteins combine the inherent strong visible fluorescent properties of GFPs with the anionic phospholipid binding specificity of annexins. Recombinant host cells, especially bacteria, are used to efficiently express the fusion proteins in high yield and soluble form, suitable for rapid, one-step affinity purification. Uses include selective cellular and biochemical labeling, particularly anionic species, such as selectively labeling apoptotic cells.		

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GFP-Annexin Fusion Proteins

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INTRODUCTION

Field of the Invention

The field of the invention is fluorescently-labeled proteins which specifically bind certain phospholipids.

Background

The annexins are a family of proteins that specifically bind anionic phospholipids, including phosphatidylserine, in a calcium-dependent manner (Blackwood, R. A. and Ernst, J. D. (1990) *Biochem. J.* 266, 195-200; Seaton, B. A. (1995) *Annexins*, R. G. Landes, Austin, TX; *Cell Mol Life Sci*, Jun 1997;53(6), entire issue). While all annexins bind phosphatidylserine and calcium, they vary in their affinity for phosphatidylserine: for example, at saturating concentrations of calcium (i.e., = 1.0 mM), annexin V exhibits a 2- to 160-fold higher affinity for phosphatidylserine compared to other members of the annexin family (Tait, J. F., et al. (1988) *Biochemistry* 27, 6268-6276; Ernst, J. D., Mall, A. and Chew, G. (1994) *Biochem Biophys Res Com* 200, 867-876). Annexin binding specificities have been exploited for biological targeting (Tait et al., 1995, *J. Biol. Chem.* 270, 21594-21599; Oshawa et al, 1996, *J. Neurochem.* 67, 89-97; Okabayashi et al., 1996, *Gene* 177, 69-76).

Apoptosis, or programmed cell death, is a universal process that is important in development of multicellular organisms, regulation of the immune system, and clearance of abnormal (including neoplastic and virus-infected) cells (Thompson, C. B. (1995) *Science* 267, 1456-62). Among the early manifestations of apoptosis in all cell types studied to date is loss of the asymmetric distribution of plasma membrane phospholipids, which results in exposure of anionic phospholipids (including phosphatidylserine) on the extracellular leaflet of the plasma membrane. This exposure of phosphatidylserine, and thus apoptosis, can be detected by various methods, including binding of labeled annexins (Koopman, G., et al.

(1994) Blood 84, 1415-20; Martin, S. J., et al. (1995) J Exp Med 182, 1545-56; Broaddus, V. C., et al. (1996) J Clin Invest 98, 2050-2059; Zhang G, et al., 1997, Biotechniques, Sep;23(3):525-531. Recently, annexin binding specificity has been correlated with other cellular pathology, e.g. King KB (1997) J Cell Biochem 65(2), 131-144. Most studies to date have used FITC-annexin V and flow cytometry to identify and enumerate apoptotic cells. Labeling annexin V with FITC requires multiple manipulations of the protein and results in a heterogeneous mixture of labeled protein molecules which vary in the number and position of bound FITC molecules. Moreover, the amino acid residue of annexin V that is most readily available for labeling by FITC is on or near the phospholipid-binding surface, which results in quenching of FITC-annexin V fluorescence by 40-50% upon binding phospholipid membranes (Tait, 1988; Ernst, 1994; supra).

In an effort to circumvent these limitations of FITC-annexins, the present inventor sought to prepare annexins that were labeled homogeneously and that did not change fluorescence properties upon binding membrane phospholipids. Described herein are the preparation and characterization of endogenously fluorescent phosphatidylserine-binding proteins containing *Aequorea victoria* green fluorescent proteins (GFPs) fused to annexins. It is shown that these reagents offer highly sensitive detection of apoptotic cells by flow cytometry or fluorescent microscopy, and offer several advantages to chemically modified annexins.

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to *Aequorea victoria* GFP-annexin fusion proteins; particularly, recombinant polypeptides comprising a bifunctional green fluorescent protein - annexin fusion protein providing an equivalent or enhanced measured fluorescent property of the green fluorescent protein and an equivalent or enhanced measured binding specificity of the annexin. In a particular embodiment, the fusion protein comprises a full-length N-terminal GFP fused to a full-length annexin V through a linker comprising an alanine, wherein the fused GFP and annexin moieties provide greater or equal fluorescent intensity and anionic phospholipid binding affinity, respectively, than do the corresponding unfused GFP and annexin proteins.

The invention also provides host cells expressing the subject proteins, including bacteria expressing the subject proteins in soluble form, and methods of using such cells to

make the fusion proteins. Uses of the subject fusion proteins include selective cellular and biochemical labeling, particularly anionic species, such as anionic phospholipids. In a particular embodiment, the fusion proteins are used to selectively label apoptotic, dead and/or injured cells.

DESCRIPTION OF PARTICULAR EMBODIMENTS OF THE INVENTION

The subject bifunctional GFP-annexin fusion proteins combine the inherent intense fluorescent properties of green fluorescent proteins with the binding specificity of annexins. The GFPs derive from the jellyfish *Aequorea victoria*; see e.g. US Patent No. 5,491,084 for definition, and include variants offering a variety of different excitation and emission wavelengths; see e.g. Heim and Tsein, 1996, Current Biology 6, 178-182. The GFP moiety of the fusion proteins provide an equivalent or enhanced measured qualitative and/or quantitative fluorescent property compared with the corresponding unfused GFP protein. Preferred fluorescent properties are emission and/or excitation peaks, preferably an maximum fluorescent emission peak in unchanged or detectably optimized wavelength and/or undiminished or enhanced in magnitude or total intensity.

The subject annexins may be derived from a variety of eukaryotic sources, see e.g. Cell Mol Life Sci, June 1997;53(6), entire issue, esp. Liemann S, Huber R, at 516-521 and Morgan RO, at 508-515, and any of the at least thirteen distinct annexin types may be used. The annexin moiety of the fusion proteins provide an equivalent or enhanced measured qualitative and/or quantitative binding specificity compared with the corresponding unfused annexin protein. Preferred binding specificities have equivalent or enhanced affinity for particular anionic cellular components, particularly phospholipids, such as phosphatidylserine.

The GFP and annexin moieties may be separated by a linker peptide, typically from about 1 to 50 residues, which facilitates or at least does not interfere with the requisite bifunctionality of the fusion proteins. The linker may enhance the conformational opportunities of the GFP and annexin moieties and/or provide a third functionality to the fusion protein, e.g. epitopes, post-translational processing sites, etc.. Exemplary linkers include alanine or polyalanine, glycine or polyglycine, epitope tags such as FLAG, processing sites such as phosphorylation, ubiquitination or protease recognition/cleavage sites, etc.

Exemplary bifunctional fusion proteins are shown in Table I.

Ref No.	GFP Variant Moiety	Annexin Moiety, residues	Linker	Activity	
				GFP	Annexin
5	T65V-I	S65T	hV (SEQ ID NO:1, residues 1-320)	ϕ	+++
	T65V-II	S65T	hV (SEQ ID NO:1, residues 12-320)	Gly	+++
	T65V-III	S65T	hV (SEQ ID NO:1, residues 3-319)	Ala	+++
	T65V-IV	S65T	mV (SEQ ID NO:2, residues 1-319)	(Ala) ₃	+++
	T65V-V	S65T	rV (SEQ ID NO:3, residues 12-319)	(Ala) ₉	+++
10	T65IV-I	S65T	hIV (SEQ ID NO:4, residues 1-321)	FLAG	+++
	T65III-I	S65T	hIII (SEQ ID NO:5, residues 1-323)	ϕ	+++
	H66V-I	Y66H	hV (SEQ ID NO:1, residues 12-320)	Gly	+++
	W66V-I	Y66W	hV (SEQ ID NO:1, residues 6-320)	(AlaGly) ₂	+++
	L64III-I	F64L	hIII (SEQ ID NO:1, residues 6-323)	Ala	+++
15	H66I-I	S65T	hI (SEQ ID NO:6, residues 1-346)	GlyAlaGly	+++
	T65I-I	S65T	hI (SEQ ID NO:6, residues 41-346)	AlaSerAla	+++

The invention provides recombinant nucleic acids encoding the subject fusion proteins. Typically, natural isolated nucleic acids encoding the GFP and annexin moieties are spliced into expression constructs using conventional methodologies, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel; *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) and references cited herein. Alternatively, the amino acid sequences of the subject peptides are used to back-translate peptide-encoding nucleic acids optimized for selected expression systems (Holler et al. (1993) Gene 136, 323-328; Martin et al. (1995) Gene 154, 150-166). In either instance, the constructs are designed for expression in any conventional system, such as bacterial, insect, plant and mammalian expression systems. The proteins are preferably secreted and/or expressed in soluble form; preferably most of the protein secreted by or retained within the host cell is in soluble form. Preferred soluble expression avoids denaturation/renaturation and permits single step affinity purification of >90%, preferably >95%, preferably in a yield of at least 10, more preferably at least 25 mg/L. In a particular embodiment, the temperature of the expressing host is reduced at least

5, preferably at least 10, more preferably at least 15°C below physiological or environmental temperature for the host (e.g. below 37°C for E.coli or human cells).

Uses of the subject fusion proteins include selective cellular and biochemical labeling, particularly anionic species, such as anionic phospholipids. The subject proteins may be exposed to the targeted cellular or biochemical component in any convenient way, e.g. direct exogenous addition, indirectly by introduction into a cell and expression of a fusion protein encoding nucleic acid, etc., In a particular embodiment, the fusion proteins are used to selectively label apoptotic, dead and/or injured cells.

Without further description, one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art. All publications and patent applications cited in this specification, and cited references therein, are herein incorporated by reference as if each individual publication, patent application or reference were specifically and individually indicated to be incorporated by reference.

EXAMPLES

Plasmid construction. Parental annexin plasmids for construction of GFP annexin fusions were constructed as previously described in published literature (e.g. Seaton, 1995, supra and citations therein). For example, the parent plasmid for construction of the GFP-annexin V fusion was pET9dE2, which were previously constructed and used for expression of human annexin V (Ernst, 1994, supra). Similarly, constructs encoding a panel of GFPs are constructed using commercially available and/or published material and methods (e.g. US Patent No. 5,491,084; Clontech, Apr 1997; Kaln, 1997, Biotechnol. International 8/97; Heim and Tsien, 1996, Current Biology 6, 178-182). For example, the open reading frame of green fluorescent protein (S65T variant) was amplified by PCR using pS65T-C1 (Clontech) as template and primers designed to incorporate sites for Rca I for ligation to the Nco I site of pET9dE2. In this case, the forward primer introduces a change in codon 2 to encode serine (as in wild-type GFP) rather than the glycine encoded by the template plasmid; and the backward primer eliminates the stop codon at the 3' end of the GFP open reading

frame and introduces an alanine codon to form the junction with the 5' end of annexin V. After digestion of the PCR product with Rca I, it was ligated to Nco I-digested pET9dE2 and used to transform *E. coli* DH5 α , and transformants that contained the GFP fragment in the correct orientation were used to transform *E. coli* BL-21(DE3) for protein expression.

5 *Expression and purification of protein.* Initial attempts at protein expression under standard conditions (growth at 37°C, induction with 0.4 mM IPTG) yielded brightly fluorescent but insoluble protein. Consequently, procedures were devised for expression of soluble, bifunctional proteins. In one embodiment, the GFP-annexin fusion proteins were expressed in *E. coli* BL-21(DE3) by growth in LB media at room temperature for 18-20
10 hours, without IPTG induction. This resulted in fusion proteins that were bright green and present in the soluble fraction (approximately 80%) after lysozyme digestion and probe sonication of the bacterial suspension. After adding calcium (2.5 mM) to the supernatant fraction of the *E. coli* lysate, GFP-annexins were purified by affinity chromatography using phospholipids (e.g. phosphatidylserine) immobilized on controlled-pore glass (Ernst, J. D.,
15 et al., (1991) J. Biol. Chem. 266, 6670-6673; Ernst, J. D. (1991) J. Immunol. 146, 3110-3114; Ernst, 1994).

20 *Characterization of GFP-annexin.* Fluorescence excitation and emission spectra were obtained using an SLM8000C spectrofluorimeter equipped with motorized excitation and emission monochromators and bandpass settings of 4 nm. Phospholipid-containing liposomes were prepared as previously described (Ernst, 1994, supra).

25 *Studies of apoptotic cells.* Rabbit and human pleural mesothelial cells were plated in 6 well plates at near confluency. For the experiments detailed below, cells were incubated overnight in the apoptotic stimuli, crocidolite (5-20 $\mu\text{g}/\text{cm}^2$) or actinomycin D (0.33 μM), in serum-free conditions to avoid serum coating onto the asbestos fiber. After floating cells
30 were collected by aspiration of medium, attached cells were detached with trypsin (2.5%) and added to the floating cells. Cells were kept on ice to minimize ongoing apoptosis. Cells exposed to asbestos fibers were filtered through a 100 μm cell strainer to remove fibers prior to flow cytometric analysis. Cells (approximately $2-5 \times 10^5$ cells per condition) were then spun and resuspended in 200 μl of annexin buffer (Hank's buffer with 15 mM Hepes and 2 mM total calcium concentration [1.3 mM in the Hank's plus an additional 0.7 mM CaCl_2]).

Cells ($2-5 \times 10^5$ in 200 μl) were incubated with GFP-annexin or FITC-annexin (both at 3 $\mu\text{g}/\text{ml}$) for 10 min on ice. Immediately prior to analysis by flow cytometry, propidium

iodide (15 μ g/ml, Sigma Chemical Co.) was added to each tube. No further washing and no fixation was performed.

Cells on ice were analysed by flow cytometry (FACSort, Becton Dickinson, San Jose, CA) with acquisition and data analysis using CELLQuest Software (Becton Dickinson). Compensation for the use of two fluorescent probes was set using control cells stained with either GFP-annexin or propidium iodide alone. 10,000 events per sample were acquired to ensure adequate mean data.

For detection of intracellular antigens, cells require fixation and permeabilization. To determine if GFP-annexin binding would be altered by those conditions, various fixatives and permeabilization were tested. During these studies, the calcium concentration was increased to 5 mM. After cells were stained with GFP-annexin as above, cells were washed twice in annexin buffer to remove all free GFP-annexin. To determine the stability of the bound GFP-annexin during fixation, the cells were then fixed with either glutaraldehyde (0.5-2%) or paraformaldehyde (2-4 %) for 10 min in the dark. The cells were then washed in annexin buffer (with 5 mM CaCl_2) and permeabilized with Triton 0.1% in the same buffer for 4 min. Following an additional wash, cells were resuspended in annexin buffer (with 5 mM CaCl_2) for analysis of GFP-annexin binding.

Expression and purification of GFP-annexins: Under the low temperature expression conditions, approximately 80% of the GFP-annexins expressed were present in the soluble fraction of the *E. coli* lysate, and generally >90% could be isolated to purity in single step phospholipid affinity chromatography. For example, GFP-annexin V proteins were isolated to >90% purity in a single step by calcium-dependent phosphatidylserine affinity chromatography. This served as an efficient purification step as well as providing evidence that the phospholipid binding ability of the annexin domains were preserved in these chimera. Another chimeric protein in which GFP was fused to the carboxyl terminus of annexin V was also soluble and fluorescent, but did not bind phosphatidylserine. The isolated proteins had the electrophoretic mobility predicted by amino acid compositions (61 kDa for annexin V fusions), were recognized by an antibody to the annexin moiety, and exhibited SDS-resistant fluorescence with UV transillumination of the gel and exhibited the functional properties of both antecedents. The failure of an annexin V fusion protein that contained the moieties in the contrasting orientation (e.g., GFP fused to the carboxyl terminus of annexin V) to bind phospholipids was unexpected and may have been the result

of the GFP domain masking the phospholipid binding surface of the annexin.

Fluorescence properties of GFP-annexins: To assure that the fusion of annexins to GFPs did not reduce the fluorescence properties of the GFP moieties, the excitation and emission spectra of the GFP-annexin fusion proteins were examined and the spectra of the GFPs found undiminished. For example, the GFP(S65T)-annexin V fusion proteins exhibited a discrete peak of fluorescence emission centered at 512 nm when excited at 490 nm. Consistent with the prior observation that the alteration of residue 65 from serine to threonine causes loss of excitation of GFP in the near ultraviolet range, there was no fluorescence detected when these GFP-annexin V chimeras were excited at 375 nm. When fluorescence emission was monitored at 510 nm, there was a single major excitation peak at 465-495 nm, with a minor trough at 480 nm. The fluorescence spectra were unaffected by the addition of calcium (1.0 mM), phosphatidylserine-containing liposomes (1.0 μ M), or both. These GFP-containing proteins are ideally suited for use with the argon-laser based flow cytometers (excitation at 488 nm/detection at 530 nm).

Use of GFP-annexin V to detect apoptotic cells by flow cytometry. To determine whether the annexin V chimeras retained the ability of annexin V to bind apoptotic cells in a specific and calcium-dependent manner, a well-characterized model system of apoptosis of pleural mesothelial cells was used (Broaddus, 1996, supra). GFP-annexin V chimeras did not bind to normal cells, but exhibited saturable binding to apoptotic mesothelial cells as detected by flow cytometry. Compared to FITC-annexin V, fluorescence of GFP-annexin V chimera-labeled apoptotic cells was 5 times brighter, despite using GFP-annexin V chimeras at approximately 1/2 the molar concentration of FITC-annexin V. In these experiments, staining with either FITC-annexin V or GFP-annexin V chimeras provided a more sensitive detection of apoptotic cells than staining with acridine orange. Binding of GFP-annexin V chimeras to apoptotic cells was calcium-dependent, and could be competitively antagonized by unlabeled annexin V. Therefore, in addition to retaining the fluorescence properties of GFP, GFP-annexin V chimeras also retain the ability of annexin V to specifically detect exposure of anionic phospholipids on the outer leaflet of the plasma membrane of apoptotic cells. GFP-annexin V chimeras could also be used in experiments in which exclusion of propidium iodide (PI) was used to distinguish early apoptotic (GFP-annexin V+, PI-) cells from late apoptotic or necrotic cells (GFP-annexin V+, PI+).

In studies of apoptosis in vivo, it may be important to identify the cell type

undergoing apoptosis. One means of phenotyping apoptosis cells is to costain with an antibody that identifies a specific cell type. Some cells, for example mesothelial cells have no unique cell surface antigen and must be identified by intracellular expression of intermediate filaments, namely cytokeratin. Access of antibodies to this or other intracellular antigens requires cell permeabilization with detergent, which disrupts the integrity of plasma membrane phospholipids. When apoptotic cells were first labeled with GFP-annexin V chimeras, washed to remove unbound fusion protein, fixed with paraformaldehyde or glutaraldehyde, and permeabilized with 0.1% Triton X-100, GFP-annexin V chimera binding was retained. Moreover, after staining with a monoclonal antibody to cytokeratin and PE-labeled goat anti-mouse IgG, two-color flow cytometry could be used to identify and enumerate apoptotic mesothelial cells in a mixture of cells (lymphocytes, macrophages, and neutrophils) obtained from the pleural space of rabbits treated with crocidolite asbestos.

Due to their bifunctional properties, GFP-annexins are useful reagents for further studies of apoptosis and of disorders of the erythrocyte membrane that are characterized by loss of phospholipid asymmetry and exposure of anionic phospholipids (Kuypers, F. A., et al. (1996) *Blood* 87, 1179-1187; Wood, B. L., Gibson, D. F. and Tait, J. F. (1996) *Blood* 88, 1873-80). In addition to the utility of GFP-annexins as reagents for studying membrane phospholipids, the approach described here is useful in constructing annexin fusions to GFP to study the interaction of specific annexins with intracellular membranes in intact cells. Annexins have been found to interact with membranes of phagosomes, endosomes, and intracellular vesicles containing microbial pathogens (Ernst, 1991, *supra*; Emans, N., et al. (1993) *J Cell Biol* 120, 1357-1369; Desjardins, M., et al. (1994) *J Biol Chem* 269, 32194-200; Majeed, M., et al. (1994) *Infect Immun* 62, 127-134; Diakonova, M., et al. (1997) *J Cell Sci* 110, 1199-213). The ability to use bifunctional GFP-annexin fusion proteins that are functionally well-characterized to study the interaction of annexins with intracellular membranes will further advance the understanding of the intracellular functions of the annexin proteins.

WHAT IS CLAIMED IS:

1. A recombinant polypeptide comprising a bifunctional *Aequorea victoria* green fluorescent protein - annexin fusion protein, said fusion protein comprising GFP and annexin moieties which provide greater or equal fluorescent intensity and anionic phospholipid binding affinity, respectively, than do the corresponding unfused GFP and annexin proteins.

2. The polypeptide of claim 1, wherein the GFP and annexin moieties are selected from: S65T GFP variant/hAnnexinV, residues 1-320 (SEQ ID NO:1, residues 1-320); S65T GFP variant/hAnnexinV, residues 12-320 (SEQ ID NO:1, residues 12-320); S65T GFP variant/hAnnexinV, residues 3-319 (SEQ ID NO:1, residues 3-319); S65T GFP variant/mAnnexinV, residues 1-319 (SEQ ID NO:2, residues 1-319); S65T GFP variant/rAnnexinV, residues 12-319 (SEQ ID NO:3, residues 1-319); S65T GFP variant/hAnnexinIV, residues 1-321 (SEQ ID NO:4, residues 1-321); S65T GFP variant/hAnnexinIII, residues 1-323 (SEQ ID NO:5, residues 1-323); Y66H GFP variant/hAnnexinV, residues 12-320 (SEQ ID NO:1, residues 12-320); Y66W GFP variant/hAnnexinV, residues 6-320 (SEQ ID NO:1, residues 6-320); F64L GFP variant/hAnnexinIII, residues 6-323 (SEQ ID NO:5, residues 6-323); S65T GFP variant/hAnnexinI, residues 1-346 (SEQ ID NO:6, residues 1-346); and S65T GFP variant/hAnnexinI, residues 41-346 (SEQ ID NO:6, residues 41-346).

3. A bacterium comprising a soluble polypeptide according to claim 1.

4. A method of making a polypeptide comprising a bifunctional green fluorescent protein - annexin fusion protein, said method comprising the steps of culturing a bacterium comprising a nucleic acid encoding a polypeptide according to claim 1 under conditions wherein the polypeptide is solubly expressed within the bacterium.

5. A method of labeling a cell comprising the step of contacting the cell with a polypeptide according to claim 1.

6. A cell which expresses the polypeptide of claim 1.

7. A mammalian cell which expresses the polypeptide of claim 1.
8. A cultured cell which expresses the polypeptide of claim 1.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁶ : C07K 19/00, 14/435, 14/47, C12N 5/10, 1/21, G01N 33/569	A3	(11) International Publication Number: WO 99/19470 (43) International Publication Date: 22 April 1999 (22.04.99)
(21) International Application Number: PCT/US98/21444 (22) International Filing Date: 9 October 1998 (09.10.98) (30) Priority Data: 08/948,276 9 October 1997 (09.10.97) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 5th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventor: ERNST, Joel, D.; University of California, San Francisco, P.O. Box 0868, San Francisco, CA 94143-0868 (US). (74) Agent: OSMAN, Richard, Aron; Science & Technology Law Group, 75 Denise Drive, Hillsborough, CA 94010 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> (88) Date of publication of the international search report: 1 July 1999 (01.07.99)
(54) Title: GFP-ANNEXIN FUSION PROTEINS (57) Abstract Bifunctional green fluorescent protein (GFP)-annexin fusion proteins combine the inherent strong visible fluorescent properties of GFPs with the anionic phospholipid binding specificity of annexins. Recombinant host cells, especially bacteria, are used to efficiently express the fusion proteins in high yield and soluble form, suitable for rapid, one-step affinity purification. Uses include selective cellular and biochemical labeling, particularly anionic species, such as selectively labeling apoptotic cells.		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21444

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K19/00 C07K14/435 C07K14/47 C12N5/10 C12N1/21
G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG D -S ET AL: "THE PLECKSTRIN HOMOLOG DOMAIN OF HUMAN BETAISIGMAII SPECTRIN IN TARGETED TO THE PLASMA MEMBRANE IN VIVO" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 225, no. 2, 14 August 1996, pages 420-426, XP002030428 see abstract	1,5-8
A	F DE GIORGI ET AL: "Targeting aequorin and green fluorescent protein to intracellular organelles" GENE, vol. 173, 1996, pages 113-117, XP002080763 see page 116, left-hand column	1,6-8



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

29 March 1999

Date of mailing of the international search report

13/04/1999

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European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Ceder, O

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/US 98/21444

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HEIM R ET AL: "ENGINEERING GREEN FLUORESCENT PROTEIN FOR IMPROVED BRIGHTNESS, LONGER WAVELENGTHS AND FLUROESCENCE RESONANCE ENERGY TRANSFER" CURRENT BIOLOGY, vol. 6, no. 2, 1 February 1996, pages 178-182, XP000676582 cited in the application see abstract; table 1 ---	2
A	WO 95 27903 A (INSTITUUT BIOPRIME ;REUTELINGSPERGER CHRISTIAAN PE (NL)) 19 October 1995 see abstract; claims 4,9 ---	5
A	WO 92 19279 A (UNIV WASHINGTON) 12 November 1992 see abstract; claims 3,23 ---	1,4
A	PEPINSKY ET AL.: "Annexin V - human" PIR1 SEQUENCE DATA BASE, 30 June 1992, XP002097363 Accession Number: Aqhup see the whole document & PEPINSKY ET AL.: "Five distinct calcium and phospholipid binding proteins share homolgy with lipocortin I" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, 1988, pages 10799-10811, ---	2
A	RODRIGUEZ-GARCIA ET AL.: "Mouse annexin V" SWISSPROT SEQUENCE DATA BASE, 1 February 1996, XP002097364 Accession Number: Anx5_Mouse see the whole document & RODRIGUEZ-GARCIA ET AL.: GENOMICS, vol. 31, 1996, pages 151-157, ---	2
A	PEPINSKY ET AL.: "Rat annexin V" SWISSPROT SEQUENCE DATA BASE, 1 April 1990, XP002097365 Accession Number: Anx5_Rat see the whole document & PEPINSKY ET AL.: "Five distinct calcium and phospholipid binding proteins share homolgy with lipocortin I" JOURNAL OF BIOCHEMICAL CHEMISTRY, vol. 263, 1988, pages 10799-10811, ---	2
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21444

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>TAIT ET AL.: "Human annexin IV" PIR1 SEQUENCE DATA BASE, 30 January 1993, XP002097366 Accession Number: A42077 see the whole document & TAIT ET AL.: "Chromosomal mapping of the human annexin IV (ANX4) gene" GENOMICS, vol. 12, 1992, pages 313-318,</p> <p>---</p>	2
A	<p>PEPINSKY ET AL.: "Human annexin III" SWISSPROT SEQUENCE DATA BASE, 1 October 1989, XP002097367 Accession Number: Anx3_Human see the whole document & PEPINSKY ET AL.: "Five distinct calcium and phospholipid binding proteins share homolgy with lipocortin I" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, 1988, pages 10799-10811,</p> <p>---</p>	2
A	<p>WALLNER ET AL.: "Human annexin I" PIR1 SEQUENCE DATA BASE, 4 December 1986, XP002097368 Accession Number: Luhu see the whole document & WALLNER ET AL.: "Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity" NATURE, vol. 320, 1986, pages 77-81,</p> <p>---</p>	2
P,X	<p>EBERHARD ET AL.: "Characterization of annexin II localization in glioma cells using green fluorescent protein-annexin chimeras" MOLECULAR BIOLOGY OF THE CELL, vol. 8 (Suppl), November 1997, page 293A XP002098187</p>	1,6-8
A	<p>see abstract</p> <p>---</p>	2
P,X	<p>ERNST ET AL.: "Preparation and characterization of an endogenously fluorescent annexin for detection of apoptotic cells" ANALYTICAL CHEMISTRY, vol. 260, 1998, pages 18-23, XP002098188 see the whole document</p> <p>-----</p>	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/21444

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9527903	A	19-10-1995	AT 160875 T	15-12-1997
			AU 689248 B	26-03-1998
			AU 2114195 A	30-10-1995
			CA 2185535 A	19-10-1995
			DE 69501161 D	15-01-1998
			DE 69501161 T	19-03-1998
			EP 0755516 A	29-01-1997
			ES 2112057 T	16-03-1998
			JP 2824155 B	11-11-1998
			JP 9505894 T	10-06-1997
			NO 964308 A	11-12-1996
			NZ 283171 A	26-02-1998
			US 5834196 A	10-11-1998
WO 9219279	A	12-11-1992	CA 2086437 A	10-11-1992
			EP 0538459 A	28-04-1993
			US 5632986 A	27-05-1997

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